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Accessory Proteins Bind a Primed Template and Mediate Rapid Cycling of DNA Polymerase III Holoenzyme from *Escherichia coli**

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DNA polymerase III holoenzyme was assembled from pure proteins onto a primer template scaffold. The assembly process could be divided into two stages. In the time-consuming first stage, β subunit and $\gamma\cdot\delta$ subunit complex were required in forming a tightly bound ATP-activated "preinitiation complex" with a single-stranded DNA bacteriophage circle uniquely primed with a synthetic pentadecadeoxyribonucleotide. This finding substantiates an earlier study using crude protein preparations in a homopolymer system lacking *Escherichia coli* single-stranded DNA binding protein (Wickner, S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 3511–3515). In the second stage, the polymerase III core and the τ subunit rapidly seek out and bind the preinitiation complex to form DNA polymerase III holoenzyme capable of rapid and entirely processive replication of the circular DNA. ATP is not required beyond formation of the preinitiation complex. It is remarkable that the fully assembled DNA polymerase III holoenzyme is so stably bound to the primed DNA circle (4-min half-time of dissociation), yet upon completing a round of synthesis the polymerase cycles within 10 s to a new preinitiation complex on a challenge primed DNA circle. Efficient polymerase cycling only occurred when challenge primed DNA was endowed with a preinitiation complex implying that cycling is mediated by a polymerase subassembly which dissociates from its accessory proteins and associates with a new preinitiation complex. These subunit dynamics suggest mechanisms for polymerase cycling on the lagging strand of replication forks in a growing chromosome.

DNA polymerase III holoenzyme, a multiprotein complex, is the replicase of *Escherichia coli* (1, 2). pol III holoenzyme[1] is composed of at least seven different subunits; four are accessory proteins (β, γ, δ, τ), and three others are tightly bound in a particle called pol III core: α (DNA polymerase), ϵ (3′,5′-exonuclease), and θ (2, 3). Pol III core, like DNA polymerase I, has only low processivity (10–20 nucleotides) (4) and catalytic activity (20 nucleotides/s/molecule) (5). Pol III holoenzyme can be reconstituted upon mixing pol III core and purified preparations of accessory proteins (2, 5, 6). The accessory proteins, τ, γ, δ, and β, collectively confer special properties on the holoenzyme which distinguish it as a replicative polymerase. Thus pol III holoenzyme rapidly locates a primer terminus ($k_{on} > 10^8$ M^{-1} s^{-1}),[2] hydrolyzes ATP upon forming a stable initiation complex with a primed template (6–9), has great catalytic efficiency on SSB "coated" ssDNA (500 nucleotides/s/molecule) (8, 10), is extremely processive in synthesis (>5000 nucleotides) (4), replicates a stretch of ssDNA to the last nucleotide (11), and then diffuses over duplex DNA in search of a new primer terminus (10). These properties are undoubtedly useful to a replicative polymerase in solving the problems encountered in replicating a duplex chromosome.

The responsibility of pol III holoenzyme in duplicating genetic information is more fully appreciated upon considering the dynamics of a polymerase at a moving replication fork. Rapidly growing *E. coli* cells dividing within 30 min initiate new rounds of replication before the parental chromosome is completed (1). This "dichotomous" mode of replication generates six replication forks in one cell; two forks are initiated at the unique bidirectional origin of replication on the parental chromosome, and four other forks result from bidirectional firing of the two daughter origins before full duplication of the parental chromosome (1). Providing a polymerase molecule each for the leading strand and the lagging strand, 12 pol III holoenzyme molecules are in action at one time. This amount is in keeping with estimates of 10–20 molecules of pol III holoenzyme/cell based on polymerase activity and Western blot analysis (using antibody to α) of cell lysates (12, 13). The direction of replication on the lagging strand is opposite that of fork movement, and synthesis is repeatedly primed with RNA primers (1). The lagging strand fragments (Okazaki fragments) produced upon extending these primers are 1–2 kb in length (1). The speed of a chromosomal replication fork in an *E. coli* cell is approximately 1 kb/s at 37 °C (14), and, therefore, the lagging strand must be primed and extended every 1 or 2 s. Since there are so few molecules of pol III holoenzyme/cell, the polymerase on the lagging strand must repeatedly cycle from a completed Okazaki fragment to a new RNA primer back at the fork. However, pol III holoenzyme in a purified state *in vitro* takes several minutes to cycle from a completed DNA template to a new primed DNA molecule (9, 10, 15–17). This polymerase cycling time is far too slow to account for the anticipated high frequency of nascent chain initiations at the replicating fork of the *E. coli* chromosome.

In this report, pure subunits are used to study the assembly of pol III holoenzyme onto a primed bacteriophage ssDNA circular chromosome coated with SSB protein. The results

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[1] The abbreviations used are: pol III holoenzyme, DNA polymerase III holoenzyme; naturally purified pol III holoenzyme, DNA polymerase III holoenzyme purified as a complex from a single source of cells; pol III core, DNA polymerase III core; [α-^{32}P]dNTPs, mixture of 60 μM each dCTP, dGTP, dATP, and 20 μM [α-^{32}P]dTTp; dAMP-PNP, 2′-deoxy-5′-adenyl imidodiphosphate; SSB, *E. coli* single-stranded DNA binding protein; RFII, circular duplex with a nick in one strand; ss, single-stranded; ϕX, bacteriophage ϕX174; M13mp18, bacteriophage M13mp18; kb, kilobase(s).

[2] k_{on} was calculated from the data of Fig. 2 in Ref. 8.

show that β and $\gamma \cdot \delta$ without any other subunits form a tightly bound ATP-activated "preinitiation complex" with the primed ssDNA circle. This result is consistent with a previous study by Wickner (6) using partially purified proteins and a homopolymer template. Preinitiation complexes on primed ssDNA circles serve as an efficient substrate for the pol III core and promote rapid cycling of polymerase from completed templates to new primed ssDNA circles that are endowed with a preinitiation complex, a useful property anticipated of a polymerase which must rapidly cycle to new lagging strand primers at a replication fork.

EXPERIMENTAL PROCEDURES

Materials—Sources were: unlabeled and labeled nucleotides, Pharmacia Biotechnology, Inc. and Du Pont-New England Nuclear, respectively; dAMP-PNP, gift of Dr. B. Alberts (San Francisco); DNA oligonucleotides, prepared as described (10); Bio-Gel A-5m, Bio-Rad; Type 2 ATP-agarose (N^6-linked), Pharmacia Biotechnology, Inc.; bovine serum albumin (Pentex V), Miles Laboratories; ϕX, M13mp18, and M13G*ori*1, prepared as described (18); an 8.4-kb derivative of M13mp18 ssDNA was a gift of Dr. Erik Falck-Pedersen (this department); SSB (98% pure), primase (97% pure), and β (90% pure), gifts of Dr. A. Kornberg (Stanford); pol III holoenzyme Fraction V (50% pure), prepared as described (2); pol III core Fraction VII (97% pure) prepared as described (19); τ (95% pure) and $\gamma \cdot \delta$ (90% pure), prepared[3] essentially as described (5) except an ATP-agarose column was added as the last step in both preparations. The concentrations of SSB and β were calculated from their absorbance at 280 nm using extinction coefficients of 1.5 ml mg^{-1} cm^{-1} (20) and 17,900 M^{-1} cm^{-1} (21), respectively. Concentration of all other proteins was determined by a modification of the method of Bradford with bovine serum albumin as a standard (22). Buffer A is 20 mM Tris-Cl (pH 7.5), 8 mM $MgCl_2$, 4% glycerol, 0.1 mM EDTA, 40 μg/ml bovine serum albumin, and 5 mM dithiothreitol. Buffer B is 20 mM Tris-Cl (pH 7.5), 40 mM NaCl, 8 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 8% glycerol, 5 mM dithiothreitol.

Primed DNAs—ϕX and M13mp18 viral ssDNA were singly primed by annealing a unique synthetic DNA oligonucleotide (15-mers 1 and 6, respectively (10)). Hybridization was in 10 mM Tris-Cl (pH 8.0), 0.3 M NaCl, and 30 mM sodium citrate (final pH 8.5) at a concentration of approximately 1 mg/ml viral ssDNA and 60 μg/ml oligonucleotide (1:20 molar ratio of circles to 15-mer). Annealing was initiated at 95 °C and allowed to cool to room temperature over 30 min. M13G*ori*1 ssDNA was primed with an RNA primer made by primase as described (10).

Replication Reactions—Replication reactions were in buffer A at 30 °C. Proteins (1–2.5 μl) were added directly to reactions at 30 °C from protein stocks stored on ice. Nucleotides were sometimes added in stages but were always at final concentrations of 0.5 mM ATP, 60 μM each dCTP, dGTP, dATP, and 20 μM [α-^{32}P]dTTP (specific activity, 8,000–20,000 cpm/pmol). Whenever pol III core was present during preincubation with primed ssDNA, dCTP and dGTP were present to stabilize the DNA primer toward digestion by the 3′,5′-exonuclease of pol III core (10), and replication was initiated by adding dATP and [α-^{32}P]dTTP. Replication reactions were quenched with an equal volume of 1% sodium dodecyl sulfate and 40 mM EDTA. In some experiments DNA synthesis was chased with 2 mM unlabeled dTTP for 1 min before being quenched.

Product Analysis—Total incorporation of radiolabel was measured by adsorption to DE81 paper (Whatman) as described (23), then dried and counted in a liquid scintillation counter. DNA synthesis was calculated as total incorporation of dNTPs (four times dTTP incorporation). Electrophoretic analysis was in neutral 0.8% agarose gels in 90 mM Tris-borate (pH 8.3), 2.5 mM EDTA. For autoradiography, dried gels were exposed to Kodak XAR-5 x-ray film. Autoradiograms in the linear range of film response were scanned by densitometry (Helena Laboratories Quick Scan, Jr.); scan peaks were quantitated by weighing the paper. Moles of RFII products were calculated from the distribution of radioactivity in the ϕX RFII and M13mp18 RFII bands (densitometric analysis), total incorporation of radioactivity, and the size of ϕX and M13mp18 ssDNAs.

[3] S. Maki and A. Kornberg, manuscript in preparation.

RESULTS

β and $\gamma \cdot \delta$ Form a Preinitiation Complex with Primed ssDNA—Pol III holoenzyme reconstituted from purified proteins (β, τ, $\gamma \cdot \delta$, and pol III core) exhibits a lag in DNA synthesis (Fig. 1). The lag is converted to a burst in synthesis upon a 2-min preincubation of the subunits with primed ssDNA (Fig. 1, *circles*). In this behavior the reconstituted pol III holoenzyme is qualitatively similar to the naturally purified pol III holoenzyme which forms a rate-limiting initiation complex with primed ssDNA prior to rapid processive synthesis (7, 8). In fact, when pol III core and τ were omitted from preincubation and only $\gamma \cdot \delta$ and β were preincubated with the primed ssDNA there was still a quantitative burst in synthesis upon adding pol III core and τ to the preincubated mixture (Fig. 1, *triangles*). The possibility of slight contamination by polymerase in the β or $\gamma \cdot \delta$ preparations was ruled out by total lack of radioactive incorporation during the preincubation with template; complete omission of [α-^{32}P]dNTPs from the preincubation did not affect the results. Primed ϕX ssDNA which was preincubated with $\gamma \cdot \delta$, β, and τ and then purified with phenol required a second preincubation with accessory proteins for burst synthesis making it unlikely that the accessory proteins modified the primer template.

The simultaneous presence of $\gamma \cdot \delta$, β, ATP, and primed ssDNA was essential during preincubation to elicit burst synthesis upon addition of the rest of the subunits (Fig. 1). This result may be interpreted as rate-limiting formation of an ATP-activated preinitiation complex of one or a combination of γ, δ, and β subunits with the primed ssDNA. Preincubation of a mixture of subunits in the absence of primed ssDNA resulted in more rapid synthesis than addition of subunits separately suggesting partial rate-limiting forma-

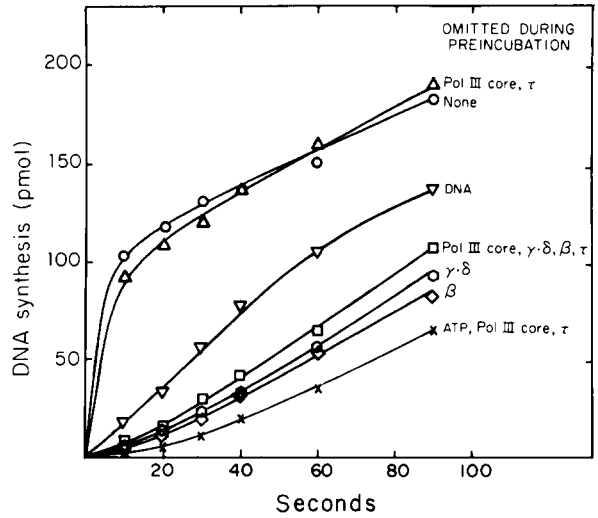

FIG. 1. **Replication time course of pol III holoenzyme reconstituted from pure subunits in two stages.** Complete replication reactions (50 μl) contained 140 ng of primed ϕX ssDNA circles (440 pmol as nucleotide), 1.4 μg of SSB, 0.5 mM ATP, 60 μM each dCTP, dGTP, dATP, and 20 μM [α-^{32}P]dTTP, 20 ng of β, 90 ng of τ, 2.6 ng of $\gamma \cdot \delta$, and 16 ng of pol III core in buffer A. Reactions were first preincubated at 30 °C for 2 min in the absence of the subunits indicated in the figure, and then replication was initiated upon addition of the rest of the subunits which had been omitted from the preincubation stage. Aliquots were removed from replication reactions at the times indicated, and DNA synthesis was quantitated as described under "Experimental Procedures." Preincubation of subunits in the absence of DNA (*upside-down triangles*) was in a volume of 7 μl of buffer A containing 8 mM $MgCl_2$ and 0.5 mM ATP. In the case where all the subunits are present in the preincubation (*circles*) DNA synthesis was initiated by adding 2 μl of 1.5 mM dATP and 0.5 mM [α-^{32}P]dTTP.

tion of a multiprotein complex prior to binding the primed template (Fig. 1). That the completely formed preinitiation complex is at the primer terminus is suggested by the inability of unprimed ssDNA (2-fold molar excess over primed DNA circles) to inhibit in these assays (not shown).

The speed and extent of the burst in DNA synthesis is similar whether pol III core and τ are preincubated with the other components (DNA, ATP, $\gamma \cdot \delta$, β) or added after the preincubation stage (Fig. 1). Hence, pol III core and τ can act at a stage after β and $\gamma \cdot \delta$ and are rapid and efficient in locating the preinitiation complex. Addition of only pol III core (without τ) to the preinitiation complex gave a burst of synthesis that was approximately one-quarter of that in the presence of τ (not shown).

The preincubation time for complete formation of the preinitiation complex was about 90 s (Fig. 2). The rate of preinitiation complex formation was not stimulated by the presence of τ, pol III core, or a mixture of τ and pol III core (not shown). Neither was the rate of preinitiation complex formation affected by using as substrate a ssDNA primed with the RNA 28-mer made by primase at the G4 origin (Fig. 2).

Both β and τ were required in excess over primed ssDNA circles for maximal synthesis in these assays (3.5 mol as β dimer ($K_d < 2$ nM) and 14 mol as τ monomer ($K_d < 8$ nM) per mol of primed ssDNA circle); pol III core was utilized at a stoichiometry of 2 core monomers/DNA circle. The $\gamma \cdot \delta$ complex was most efficient in reconstituting pol III holoenzyme; 2.5 ng of $\gamma \cdot \delta$ supported replication of 20 fmol (as circles) of primed ϕX DNA in the burst phase of DNA synthesis (Figs. 1 and 2). Assuming a molecular mass of 200 kDa (5, 7) and the protein concentration measured by Bradford reagent, only one molecule of $\gamma \cdot \delta$ was utilized in replicating 1.6 DNA circles in the 20-s burst of synthesis.

The Preinitiation Complex Physically Associates with Primed ssDNA—A template challenge experiment was designed to test for a physical association between the preinitiation complex and the primed ssDNA (Fig. 3*A*). In the template challenge assay, $\gamma \cdot \delta$ was preincubated with a 6-fold molar excess of primed ssDNA along with saturating amounts of β and τ. After preincubation for 2 min at 30 °C a challenge primed ssDNA was added, and synthesis was initiated upon adding pol III core. Although 10 s is sufficient time to replicate ϕX ssDNA (10) and 15 s is sufficient time for complete

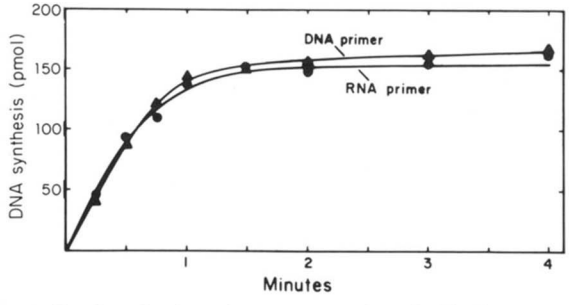

FIG. 2. **Preincubation time course of preinitiation complex formation.** Replication reactions (50 μl) contained 140 ng of primed M13G*ori*1 ssDNA circles, 1.4 μg of SSB, 0.5 mM ATP, 300 μM each CTP, GTP, and UTP, 60 μM each dCTP, dGTP, and dATP, 20 μM [α-^{32}P]dTTP, 20 ng of β, 90 ng of τ, and 2.6 ng of $\gamma \cdot \delta$ in buffer A. Reaction mixtures were preincubated at 30 °C for the indicated time before initiating synthesis upon adding 16 ng of pol III core. The samples were quenched after 20 s of replication, and DNA synthesis was quantitated as described under "Experimental Procedures." *Triangles*, M13G*ori*1 ssDNA was singly primed with a DNA 15-mer. *Circles*, M13G*ori*1 ssDNA was primed with an RNA 28-mer at the G4 origin in a prior 10-min incubation with 42 ng of primase.

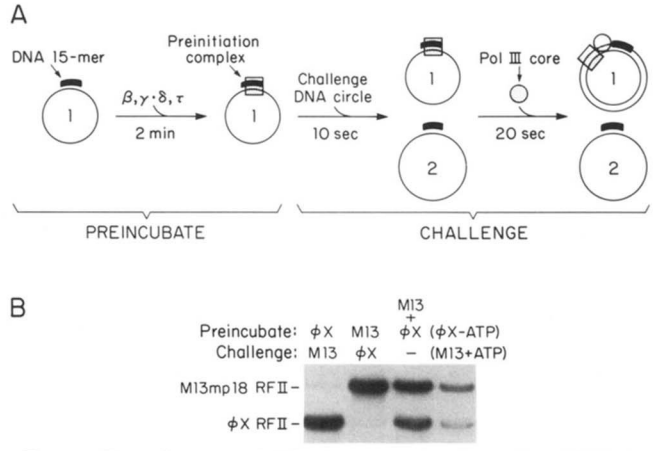

FIG. 3. **Test for a preinitiation complex bound to DNA by template challenge experiments.** *A*, scheme for performing template challenge assays. *B*, analysis of template challenge assays by native agarose gel electrophoresis. Preincubation reactions contained 0.7 μg of SSB, 0.5 mM ATP, 60 μM each dCTP and dGTP, 10 ng of β, 45 ng of τ, 1.3 ng of $\gamma \cdot \delta$, and 72 ng of primed ϕX ssDNA or primed M13mp18 ssDNA in 25 μl of buffer A. After preincubation at 30 °C for 2 min a second solution (25 μl) was added which contained 0.7 μg of SSB, 0.5 mM ATP, 60 μM each dCTP and dGTP, 120 μM dATP, 40 μM [α-^{32}P]dTTP, and 72 ng of the challenge primed bacteriophage ssDNA in buffer A. Replication was initiated upon adding 8 ng of pol III core, and 20 s later the reaction was chased with 2 mM unlabeled dTTP for 1 min and then quenched and analyzed in a native agarose gel. In *lane 3* both ϕX and M13mp18 primed ssDNAs were present in the preincubation reaction in a volume of 50 μl. In *lane 4* ATP was omitted from the preincubation reaction but was present in the solution containing challenge DNA.

replication of M13mp18 ssDNA,[4] the reactions were for 20 s and then chased with excess unlabeled dTTP for 1 min to ensure all template circles initiated with [α-^{32}P]dNTPs were taken to full-length RFII products. The template challenge assays showed replication only on the DNA template preincubated with the accessory proteins; the challenge primed ssDNA appeared untouched (Fig. 3*B*, *lanes 1* and *2*). The control preincubation of accessory proteins with a mixture of both ϕX and M13mp18 primed ssDNA circles gave the expected equal replication of both templates (Fig. 3*B*, *lane 3*). These results support the conclusion that a preinitiation complex is formed between accessory protein(s) and the primed ssDNA.

Template challenge experiments performed as in Fig. 3 but in the complete absence of τ gave the same pattern of DNA synthesis as in the presence of τ except total DNA synthesis was about 8-fold lower (data not shown). Addition of τ after preincubating β and $\gamma \cdot \delta$ with primed ϕX ssDNA stimulated synthesis as expected, yet radioactive incorporation remained exclusively on the preincubated template supporting the conclusion of Fig. 1 that τ acts at a step after preinitiation complex formation (not shown but identical to *lane 1* in Fig. 3*B*).

ATP Requiring Step in Assembly of Pol III Holoenzyme on Primed ssDNA—An ATP requirement in formation of the preinitiation complex was indicated by two criteria. First, ATP is needed during preincubation of accessory proteins with primed ssDNA for the burst in DNA synthesis (Fig. 1). Second, ATP was essential for preferential replication of preincubated ϕX DNA in a template challenge experiment (Fig. 3, *lane 4*). To test for an ATP requirement by τ or pol III core in the second stage of pol III holoenzyme assembly, primed ϕX ssDNA was preincubated with β, $\gamma \cdot \delta$, and ATP,

[4] M. O'Donnell, unpublished data.

and then preinitiation complexes were gel-filtered on Bio-Gel A-5m. This resin separates free ATP and all other components not bound to DNA in the included volume from protein·DNA complexes in the excluded fraction. Addition of pol III core and τ to the DNA in the excluded fraction gave the expected amount of burst synthesis activity, further evidence that the preinitiation complex is bound to the primed ssDNA (Table I). Approximately 75% of the DNA recovered in the excluded fraction contained a preinitiation complex (Table I, compare reactions 6 and 7). Addition of pol III core and τ to gel-filtered preinitiation complexes without ATP resulted in the same amount of replication as when ATP was added back (Table I, compare reactions 5 and 6, and also compare reactions 3 and 4). Stimulation of DNA synthesis by τ was about 3-fold both before and after gel filtration (Table I, compare reactions 1 and 2 with reactions 3 and 5, and also compare reactions 1 and 2 with reactions 4 and 6). Hence ATP is not required for association of pol III core and τ with the preinitiation complex; neither is additional β or $\gamma\cdot\delta$ required for association of pol III core and τ. In this gel filtration study dAMP-PNP was used in place of dATP in the replication reactions. dAMP-PNP is incorporated in place of dATP during replication but does not replace ATP in supporting initiation complex formation which requires hydrolysis of the β,γ-phosphoanhydride bond of ATP or dATP (8). One minute was provided for assay of the preinitiation complex since replication supported by dAMP-PNP is approximately 10-fold slower than with dATP (10). The absence of ATP or dATP contamination in the dAMP-PNP or the dCTP, dGTP, and dTTP preparations was demonstrated as described (10).

Time That the Preinitiation Complex Remains Bound to a Primer Template—The time that a preinitiation complex (with τ present in the preincubation) remains bound to a primed ϕX ssDNA was measured by template challenge assays as in Fig. 3 except the ϕX DNA and M13mp18 DNA circles were incubated together for various times before adding pol III core to initiate synthesis. The agarose gel analysis of the time course of transfer of the preinitiation complex from ϕX DNA to M13mp18 DNA is shown in Fig. 4. The distribution of radioactivity in the viral DNA products showed that the half-time for transfer of the preinitiation complex from ϕX to the M13mp18 primed ssDNA was approximately 2 min (Fig. 4). Transfer time of the preinitiation complex in the absence of τ was only slightly increased ($t_{1/2}$ transfer was approximately 3 min, not shown).

TABLE I

Lack of a further ATP requirement for assembly of pol III holoenzyme after the preinitiation complex

Preinitiation complexes were formed in buffer A (125 μl) containing 1.8 μg of primed ϕX ssDNA, 18 μg of SSB, 32 ng of $\gamma\cdot\delta$, 100 ng of β, and 0.5 mM ATP. After incubating for 2 min at 30 °C two 5-μl samples were assayed, and the remainder was chilled on ice and filtered over a 7.5-ml column of Bio-Gel A-5m equilibrated in buffer B at 4 °C. Aliquots of the excluded fraction were assayed upon adding dNTPs to a final concentration of 60 μM each dCTP and dGTP, 50 μM dAMP-PNP, and 20 μM [α-^{32}P]dTTP, 0.5 mM ATP (when present), 16 ng of pol III core, 45 ng of τ (when present), 1.3 ng of $\gamma\cdot\delta$ (when present), and 10 ng of β (when present). DNA synthesis is calculated for the entire sample loaded onto the column.

Components added	DNA synthesis
	pmol in 1 min
Before gel filtration	
1. pol III core	288
2. pol III core, τ	863
After gel filtration	
3. pol III core	167
4. pol III core, ATP	186
5. pol III core, τ	493
6. pol III core, τ, ATP	507
7. pol III core, ATP, τ, $\gamma\cdot\delta$, β	679

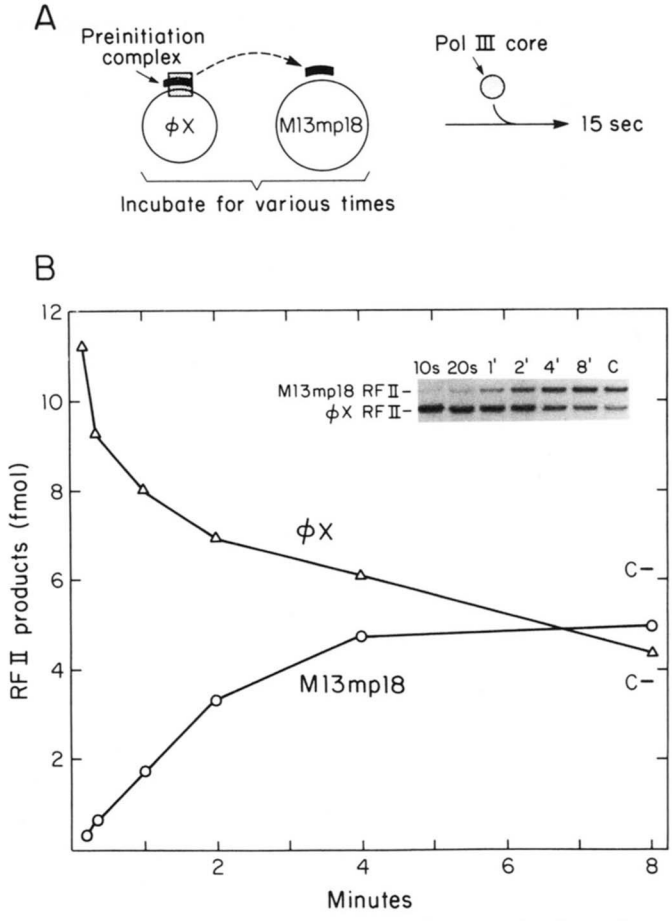

FIG. 4. **Time the preinitiation complex remains bound to a primed ssDNA circle.** *A*, scheme to measure the time of preinitiation complex transfer from one primed circle to another. *B*, time course of transfer of the preinitiation complex from ϕX DNA to M13mp18 DNA. Each time point was a separate reaction performed as follows. Singly primed ϕX ssDNA (72 ng) coated with 0.72 μg SSB was preincubated for 2 min at 30 °C with 10 ng of β, 1.3 ng of $\gamma\cdot\delta$, 45 ng of τ, 0.5 mM ATP, 60 μM each dCTP, dGTP, dATP, and 20 μM [α-^{32}P]dTTP in 25 μl of buffer A. Transfer of the preinitiation complex to M13mp18 DNA was initiated upon mixing with 25 μl of buffer A containing 72 ng of primed M13mp18 ssDNA, 0.72 μg of SSB, 60 μM each dCTP, dGTP, dATP, and 20 μM [α-^{32}P]dTTP. Transfer of the preinitiation complex was allowed to proceed at 30 °C for the time indicated before initiating replication with 32 ng of pol III core. After 15 s the reaction was chased with 2 mM unlabeled dTTP for 1 min and then quenched. Moles of RFII products were calculated for each reaction as described under "Experimental Procedures." The *dashes* marked *C* is a control reaction designed to reflect the end point of the transfer reaction (see *inset*). *Inset*, neutral agarose gel analysis of the ϕX and M13mp18 RFII products in each timed sample. The *lane* marked *C* is a control reaction where a mixture of ϕX and M13mp18 primed ssDNAs was preincubated with β, $\gamma\cdot\delta$, and τ.

Time That the Pol III Core Remains Bound to an Initiation Complex—The time that the pol III core remains bound in an initiation complex with the accessory proteins (β, $\gamma\cdot\delta$, τ) while idling on primed ssDNA with two dNTPs was measured by a template challenge experiment in which the ϕX template had a complete initiation complex (all proteins present) and the challenge M13mp18 template had a preinitiation complex (with τ present in the preincubation) (Fig. 5). The initiation complex on ϕX DNA and preinitiation complex on M13mp18 DNA were mixed together, and at various times an aliquot

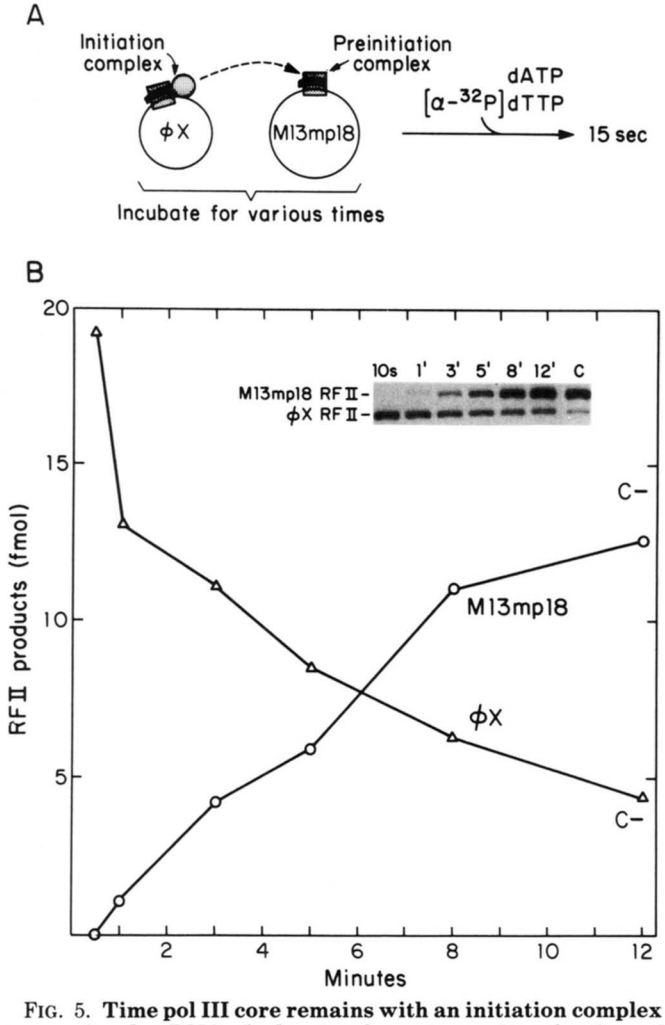

FIG. 5. **Time pol III core remains with an initiation complex on a primed ssDNA circle.** *A*, scheme to monitor the time of polymerase transfer from an initiation complex on φX DNA to a preinitiation complex on M13mp18 DNA. *B*, time course of polymerase transfer from the initiation complex on primed φX ssDNA to a preinitiation complex on primed M13mp18 ssDNA. The initiation complex on φX ssDNA was formed in a 2-min incubation (175 μl) containing 0.5 μg of primed φX ssDNA, 5 μg of SSB, 0.5 mM ATP, 60 μM each dCTP and dGTP, 70 ng of β, 315 ng of τ, 9 ng of γ·δ, and 21 ng of pol III core in buffer A. In a separate tube, the preinitiation complex on M13mp18 ssDNA was formed in a 2-min incubation (175 μl) containing 1.5 μg of primed M13mp18 ssDNA, 15 μg of SSB, 0.5 mM ATP, 60 μM each dCTP and dGTP, 70 ng of β, 315 ng of τ, and 27 ng of γ·δ in buffer A. The two preincubated reactions were mixed together, and transfer of polymerase from the φX template to preinitiation complexes on M13mp18 templates was monitored by removing samples (57 μl) of the mixture at the indicated times. As samples were collected they were immediately mixed with dATP and [α-^{32}P] dTTP (60 and 20 μM final concentrations, respectively) to initiate DNA synthesis and then chased after 15 s with 2 mM unlabeled dTTP for 1 min before being quenched. Moles of RFII products were calculated as described under "Experimental Procedures." The *dashes* marked *C* is a control reaction designed to reflect the end point of the transfer reaction (see *inset*). *Inset*, neutral agarose gel analysis of the φX and M13mp18 RFII products in each timed sample. The *lane* marked *C* is a control reaction where pol III core was added to a mixture of preinitiation complexes on φX and M13mp18 templates.

was removed, replicated for 15 s upon adding remaining dNTPs, and then chased with unlabeled dTTP for 1 min. Agarose gel analysis of the timed samples showed that the half-time for transfer of pol III core from the initiation complex on φX ssDNA to the preinitiation complex on M13mp18 ssDNA was approximately 4 min (Fig. 5). The transfer reaction in Fig. 5 only appears more complete than that in Fig. 4 due to a higher concentration of challenge template used in the reaction of Fig. 5.

Rapid Cycling of Pol III Core to New Templates during Replication—The transfer time of the reconstituted pol III holoenzyme from a completed DNA template to a challenge primed ssDNA circle was measured by mixing a 6-fold excess of M13mp18 challenge DNA and all four dNTPs with a solution containing primed φX ssDNA with a bound pol III holoenzyme fully assembled (Fig. 6*A*). Samples were withdrawn at timed intervals, quenched, and analyzed on a neutral agarose gel (Fig. 6*B*). As is true for pol III holoenzyme (9, 10, 15–17) the reconstituted pol III holoenzyme was very slow in cycling from a replicated circle (φX) to a new primed circle (M13mp18); only half the number of M13mp18 RFII products were produced in 90 s as φX RFIIs produced in the 15-s burst of synthesis (Fig. 6*C*, *left side*). In fact, there was more cycling of polymerase to the φX ssDNA than to the excess primed M18mp18 ssDNA suggesting that the polymerase is more efficient in cycling to primed DNA with a preinitiation complex then to DNA with a naked primer. In this connection, when the 6-fold excess of M13mp18 challenge template contained a preinitiation complex the constituted polymerase cycled rapidly from the φX RFII product to the excess challenge M13mp18 template (Fig. 6, *B* and *C*, *right side*). Using a value of 25 fmol of φX RFII produced in the burst phase of synthesis, the polymerase replicated φX and then cycled to and catalyzed a full round of replication on the challenge M13mp18 circle within 35 s, a second round on M13mp18 within 56 s, and yet a third round within 83 s (Fig. 6*C*, *right side*). Using a value of 10 s to replicate φX ssDNA (10) and 15 s to replicate M13mp18 ssDNA,[4] the polymerase spent 10 s in each cycling event. The naturally purified pol III holoenzyme cycled to preinitiation complexes on challenge DNA just as rapidly as the reconstituted pol III holoenzyme (not shown). That rapid polymerase cycling was mediated by preinitiation complexes rather than accessory proteins in free solution was shown by several criteria. 1) Maximum speed of polymerase cycling correlated with the level of accessory proteins required to saturate challenge DNA with preinitiation complexes. 2) Cycling of polymerase from φX DNA to a mixture of two challenge DNAs containing M13mp18 with a preinitiation complex and an 8.4-kb derivative of M13mp18 with a naked primer (same DNA 15-mer as on M13mp18) resulted in rapid polymerase cycling only to the M13mp18 DNA endowed with a preinitiation complex. 3) Preincubation of challenge DNA with accessory proteins was required for maximum speed of cycling.

DISCUSSION

The assembly of pol III holoenzyme from purified subunits was dissected into two stages (summarized in Fig. 7). In a time-consuming first stage, β and γ·δ form an ATP-activated preinitiation complex which is tightly bound to a bacteriophage ssDNA circle primed with a synthetic DNA 15-mer. In the rapid second half of pol III holoenzyme assembly, pol III core and τ locate and tightly bind to the preinitiation complex. The fully assembled pol III holoenzyme is stably bound to the primed ssDNA and in the presence of all four dNTPs rapidly replicates the entire circle. After completing replication of the ssDNA circle the polymerase cycles within 10 s to a new primed ssDNA circle but only if the new DNA circle is endowed with a preinitiation complex.

The existence of a stable ATP-activated preinitiation complex of accessory proteins(s) with primed ssDNA which required only ATP, β, and γ·δ was shown by several criteria. Only β, γ·δ, and ATP were required during preincubation

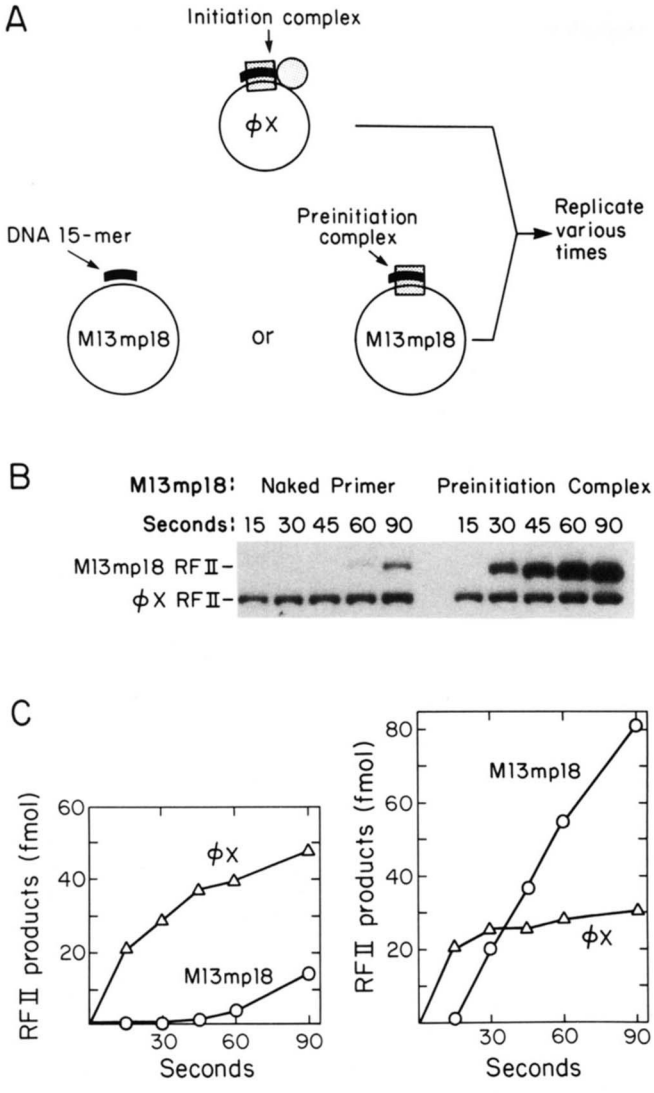

FIG. 6. **Influence of accessory proteins on the speed of polymerase cycling to new primers during replication.** *A*, scheme to study polymerase cycling from a completed template to challenge DNA with either a naked DNA primer or a preinitiation complex. *B*, neutral agarose gel analysis of polymerase cycling to challenge templates containing either a naked primer (*left*) or a preinitiation complex (*right*). Initiation complexes on ϕX DNA templates were formed in a 2-min preincubation in 150 μl of buffer A at 30 °C containing 0.42 μg of primed ϕX ssDNA, 4.2 μg of SSB, 0.5 mM ATP, 60 μM each dCTP and dGTP, 126 ng of β, 270 ng of τ, 7.8 ng of γ·δ, and 19 ng of pol III core. In a separate tube, primed M13mp18 ssDNA was incubated for 2 min at 30 °C in 150 μl of buffer A containing 2.1 μg of primed M13mp18 ssDNA, 21 μg of SSB, 126 ng of β, 270 ng of τ, 39 ng of γ·δ (when present), 0.5 mM ATP, 60 μM each dCTP and dGTP, 120 μM dATP, and 40 μM [α-^{32}P]dTTP. Replication was initiated upon mixing the preincubated ϕX template with the preincubated M13mp18 template. Samples (45 μl) were removed at the times indicated, chased for 2 min with 2 mM unlabeled dTTP, and then quenched and analyzed in a neutral agarose gel. *Left*, γ·δ was not included in the preincubation of the M13mp18 template. *Right*, γ·δ was included in the preincubation with the M13mp18 template. *C*, densitometric analysis of the autoradiograms in part *B*. *Left*, γ·δ was not included in the preincubation of the M13mp18 template. *Right*, γ·δ was included in the preincubation with the M13mp18 template. Moles of RFII products were calculated as described under "Experimental Procedures."

with primed ssDNA for a burst of synthesis upon adding pol III core and τ (Fig. 1). The preinitiation complex required minutes to dissociate from primed ssDNA (Fig. 4), was stable to gel filtration (Table I), and resisted challenge by another primed template (Fig. 3). These results substantiate a much earlier study using partially purified proteins in a homopolymer system lacking SSB and τ (6). In the earlier study crude preparations of dnaZ protein (correlates with γ by molecular weight, *N*-ethylmaleimide sensitivity, and ability to complement an extract of dnaZts cells (24)) and elongation factor III (probably δ (24)) formed a 200-kDa complex (6) which together with elongation factor I (correlates with β by molecular weight and *N*-ethylmaleimide resistance (24)) utilized ATP to bind tightly to a poly(dA) template primed with oligo(dT) (6). In the previous study only elongation factor I (β) appeared to be bound to the template suggesting that the dnaZ protein·elongation factor III complex (γ·δ) was only required catalytically to transfer elongation factor I (β) to the template; however, the method of detection of the various proteins bound to the template was indirect (6). A direct determination of the identity and stoichiometry of subunits in the preinitiation complex will require further study.

The naturally purified pol III holoenzyme attains its tight ATP-activated grip onto a primer template within a few seconds (8). In contrast, β and γ·δ when added separately to a reaction required a full minute to bind completely to the primed ssDNA (Fig. 2). It would appear that rapid binding to a primed template requires prior formation of a multisubunit complex such as a complex of γ·δ, β, and ATP (and possibly other subunits). Study of the rate of primer binding by preincubating various combinations of subunits before adding them to primed ssDNA is hampered by the low concentrations of the pol III core preparation (10 μg/ml) and γ·δ preparation (2 μg/ml). Nevertheless, preincubation of a mixture of τ, β, and the dilute pol III core and γ·δ preparations before adding them to primed ssDNA decreased the lag in synthesis by about half (Fig. 1) suggesting that formation of a multiprotein complex may precede the primer binding step. More concentrated protein stocks are being prepared in order to extend these studies. A protein(s) in the naturally purified pol III holoenzyme preparation that accelerates the primer acquisition step by pol III holoenzyme but is missing from the highly purified reconstitution system must also be considered.

In the second half of pol III holoenzyme assembly, pol III core and τ rapidly associated with the preinitiation complex on primed ssDNA resulting in fast and processive synthesis (Fig. 1) without a further ATP requirement (Table I). That τ stimulates synthesis after the preinitiation complex has formed indicates a role for τ in the speed or processivity of pol III holoenzyme which may be the result of a more intact form of pol III holoenzyme (*i.e.* containing a dimeric pol III core (25)). Pol III core is stable for minutes when bound to the preinitiation complex (Fig. 5). Yet upon completing synthesis of a circle the polymerase cycles with 10 s to a fresh preinitiation complex on another primed circle (Fig. 6*B*). Cycling of polymerase to a new preinitiation complex is about 20 times faster than cycling of pol III holoenzyme to a circle with a naked primer (Fig. 6*B*). The requirement for a bound preinitiation complex on challenge DNA circles for rapid cycling of polymerase to a challenge template suggests that cycling is mediated by a polymerase subassembly such as pol III core or pol III′ (a complex of pol III core and τ (25)). In this model, upon completing DNA synthesis, the polymerase subassembly dissociates from its accessory proteins in order to reassociate with a new preinitiation complex. The hypothesis that only a subassembly of pol III holoenzyme cycles to new templates is supported by a recent observation of tight binding of [^{3}H]β to pol III holoenzyme while in the initiation complex but complete exchange of the [^{3}H]β with the excess

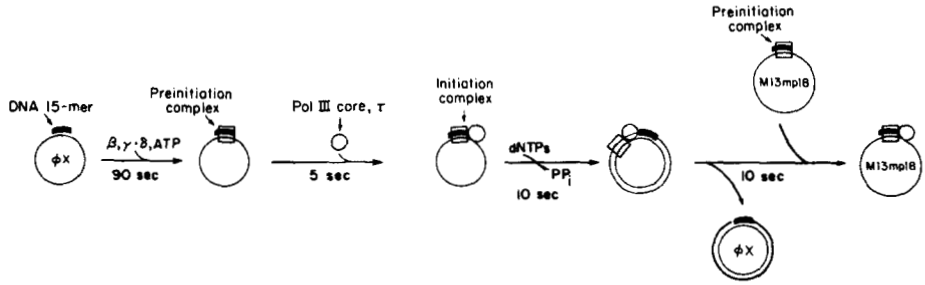

FIG. 7. **Dynamics of pol III holoenzyme subunits in replicating primed ssDNA circles.**

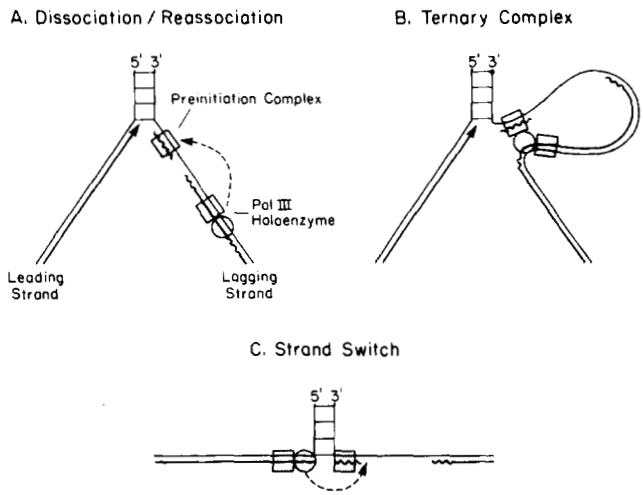

FIG. 8. **Schemes for polymerase cycling to lagging strand primers at a chromosomal replication fork.** Models of DNA polymerase cycling to a preinitiation complex on a lagging strand RNA primer. *A*, dissociation/reassociation on the lagging strand. *B*, transfer via a ternary complex between one polymerase and two preinitiation complexes on the lagging strand. *C*, dissociation/reassociation in switching from the leading strand to the lagging strand.

unlabeled β in free solution upon cycling of pol III holoenzyme to another template (17).

The subunit dynamics described here suggest a model for pol III holoenzyme in cycling to multiple primers on the lagging strand of a growing chromosome (Fig. 8). In the model of Fig. 8, a preinitiation complex forms on an upstream (anti-elongation direction) lagging strand primer while a downstream Okazaki fragment is being extended by pol III holoenzyme. Upon completing the Okazaki fragment to a nick a polymerase subassembly such as pol III core or pol III′ rapidly cycles to the new preinitiation complex on an upstream primer possibly by a dissociation/reassociation path (Fig. 8*A*) or by directly transferring via a ternary complex (Fig. 8*B*). Strand switching of polymerase among preinitiation complexes on the leading and lagging strands can also be entertained (Fig. 8*C*). Consistent with these hypotheses is the cellular abundance of β (300 molecules/cell (26)) relative to the low level of pol III holoenzyme (10–20 molecules/cell (12, 13)). The observed 10 s for polymerase cycling in this study at 30 °C approaches the 1 cycle/s needed for lagging strand synthesis *in vivo* at 37 °C (14). Possibly, the proximity of intramolecular primers on the lagging strand *in vivo* and the influence of neighboring proteins at the replication fork may speed up the efficiency of polymerase cycling.

The dynamics of the pol III holoenzyme accessory proteins share similarities with the T4 DNA replication machinery. In the T4 replication system three T4 polymerase accessory proteins hydrolyze ATP (27), confer processivity to the T4 DNA polymerase (27), and bind a primer terminus in the absence of T4 DNA polymerase (28). It is hypothesized that the T4 DNA polymerase holoenzyme uses the energy of ATP hydrolysis to displace the polymerase subunit from the primer terminus upon being constrained to idle at a primer terminus (only 3 dNTPs present) or upon completing replication (28). Likewise, the accessory proteins of pol III holoenzyme utilize ATP (Figs. 1 and 3), confer processivity to the pol III core (4, 29), and bind a primed template (Fig. 3) (6). Although the accessory proteins do not displace the idling pol III core from a primer terminus (Fig. 5) they do promote cycling (dissociation) of polymerase from a completely extended DNA chain (Fig. 6).

Besides mediating polymerase cycling, the tightly bound preinitiation complex is probably the main actor in the tight grip of pol III holoenzyme to primed ssDNA bringing us one step closer to understanding the basic principles of processivity. The structure of the preinitiation complex, the exact mechanism by which the accessory proteins mediate cycling of the polymerase and how they interface with priming proteins must await more detailed studies of the replication machinery.

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